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DOI:
10.1016/S0146-6380(02)00186-9

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Collins, MJ, Walton, D, Curry, GB, Riley, M, Von Wallmenich, TN, Savage, NM, Muyzer, G & Woestbroek, P 2003, 'Long-term trends in the survival of immunological epitopes entombed in fossil brachiopod skeletons', *Organic Geochemistry*, vol. 34, pp. 89-96. https://doi.org/10.1016/S0146-6380(02)00186-9

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Organic Geochemistry 34 (2003) 89–96
Organic Geochemistry
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Long-term trends in the survival of immunological epitopes entombed in fossil brachiopod skeletons

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Received 14 April 2002; accepted 11 September 2002
(returned to author for revision 29 May 2002)

Abstract

We report the most comprehensive study of survival of peptide bonds and epitopes (antibody binding sites) in fossil shells from a semi-continuous New Zealand brachiopod sequence extending for 3 Ma. The study reveals for the first time long-term trends in proteins survival. The investigation focused on a sub-set of the total skeletal biomolecules, those protected from exposure to a strong oxidising agent (NaOCl); the so-called intra-crystalline component. The extent of peptide bond hydrolysis was compared with the declining immunological signal. The proportion of free amino acids increased very rapidly but between 5 and 10% of the amino acids remained peptide bound in all samples. The pattern of loss of immunological reactivity broadly mirrored the loss of peptide bonds, but overall loss of signal was much greater. Significant antibody response was observed in some but not all late Pliocene fossils (>3 Ma), but against a panel of antisera the pattern of reactivity was lost in samples >0.5 Ma. Alternative models of polypeptide chain scission were used in an to attempt to relate the rate of peptide bond hydrolysis to the loss of immunological determinants. The findings suggest that, despite early optimistic reports, the application of immunology to shell carbonates does not appear capable of extending into deep time.
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1. Introduction

Despite much interest and investigation of the exceptional stability of ancient proteins, there have been no systematic studies of the loss of immunological epitopes or protein decomposition over geological time. This is perhaps not surprising, as the fate of proteins in the archaeological and geological records has much to do with the localised diagenetic environment. Most proteins are rapidly degraded, and recovery of ancient proteins is usually from exceptional environments: in cold or frozen settings, or in phases protected from the most corrosive of chemicals, water (e.g. tar pits, amber; Collins et al., 1998).

In the absence of long-term patterns of diagenesis there remains an apparent dichotomy between rapid loss of immunological signals reported in the forensic

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PII: S0146-6380(02)00186-9

literature (e.g. Quarino and Koblenski, 1988) and also in burial experiments (Cattaneo et al., 1992), and claims of long-term preservation (e.g. Muyzer et al., 1992a; Schweitzer et al., 1997; Gibert et al., 1998).

Exceptional environments by definition preclude systematic surveys of long-term survival; in order to investigate long-term trends one has to find a commonplace environment with 'exceptional' preservation. We have previously argued that brachiopod shells, despite their relative scarcity in Recent sediments, provide a stable environment for long-term preservation because of their mineralogy and ultra-structure. In most extant brachiopods the shell is composed primarily of long fibres of low magnesium calcite (MacKinnon and Williams, 1974) which are readily isolated in modern shells (Collins et al., 1988).

The organic matrix contains proteins (Collins et al., 1991a) and lipids (Stern et al., 1999), some of which survive prolonged exposure of the fibres to a strong oxidising agent such as NaOCl. Demineralization of bleach treated crystallites yields an organic fraction (Walton, 1998), the so-called 'intra-crystalline' extract (Sykes et al., 1995; Collins and Riley, 2000). The intra-crystalline extract from brachiopod shells arguably represents a commonplace environment for 'exceptional' preservation; organic matter entrapped within a diagenetically stable mineral phase.

In order to follow long-term trends in diagenesis it is necessary to assemble long sequences of well-dated shells that have all experienced similar integrated thermal histories. A suitable set of brachiopod shells has been collected from the thick Plio-Pleistocene marine sequences of the Wanganui Basin in North Island, New Zealand (Fleming, 1953) where biostratigraphic, aminostratigraphic, magnetostratigraphic, and fission-track dating provide a strong chronological framework (Bowen et al., 1998). The active tectonism that has elevated and exposed the shallow marine deposits results from subduction of the Pacific plate below North Island. The enhanced geothermal gradient in this region has the potential to significantly enhance the rate of diagenesis, but seismic evidence (Anderton, 1981) suggests that none of the sampled horizons have been buried by more than about 1 km.

We raised antisera against the total intra-crystalline organic fraction of extant brachiopods and used them to compile a serotaxonmic classification (Endo et al., 1994; but see Cohen, 1995; Marin et al., 1999). Using concentrated extracts we were able to demonstrate that the pattern of reactivity from Modern samples was also seen in the Pleistocene brachiopods from New Zealand (Collins et al., 1991b), although the limited number of samples prevented us from investigating the pattern of diagenesis.

A comprehensive analysis of the amino acid composition of the intra-crystalline fraction has recently been completed on four brachiopod genera at 18 horizons within the Wanganui Basin spanning 3 million years (Walton, 1998).

The aim of the present study is to combine the two approaches and hence contrast the loss of amino acids from the intra-crystalline fraction with the pattern of loss of the immunological signal in these New Zealand shells.

2. Materials and methods

2.1. Sample collection

Recent samples were collected from localities given in Endo et al. (1994). Fossil brachiopods (*Neothyris* sp., *Calloria* sp., *Terebratella* sp. and *Notosaria* sp.) were collected in New Zealand from the localities given in Fleming (1953), Walton et al. (1993) and Walton (1998), and prepared according to the methods previously reported (Walton and Curry, 1994).

2.2. Sample preparation

Details of sample preparation are given elsewhere (Walton, 1998). Briefly, shells were surface cleaned by soaking in an aqueous solution of NaOCl (10% vol./vol.) for 2 h at room temperature, rinsed in Milli RO water and air-dried. Samples were then ground and the powder incubated in an aqueous solution of NaOCl (10% vol./vol.) under constant motion for 24 h at room temperature, washed by repeated agitation with Milli-Q water and centrifuged (typically 10 washes) and lyophilised. For amino acid analyses an aqueous solution of 2 M HCl at a ratio of 11 μl/mg was used, and the supernatants cleared by centrifugation (20 g.h.). For immunoanalysis samples were demineralized in 20% wt./vol. ethylenediaminetetraacetic acid (EDTA) and these crude extracts were used without further purification.

2.3. Artificial diagenesis experiments

Isolated secondary layer fibres were sealed in glass tubes and heated at 140, 120 and 90 °C for up to 2 weeks. Individual tubes were opened, the fibres were demineralised and analysed for the loss of immunological reactivity as described below.

2.4. Immunoanalysis

Immunoanalysis was carried out as reported previously (Collins et al., 1991c). 100 μl of EDTA extract

(representing <11 mg of skeletal carbonate) was incubated in plastic microtitre plates. Polyclonal antibodies previously raised against skeletal macromolecules (Collins et al., 1991c) were added in doubling dilution. The mean value of the dilutions that fell in the log-linear regions of the dilution curves is used. EDTA extracts of a range of fossil molluscs (gastropods and bivalves) from the same fossil sequence were used as negative controls.

3. Results

3.1. Immunology

The pattern of loss of immunological signal displays a very rapid initial decline and a long persistent tail (Fig. 1). The patterns were similar for all four genera despite differences in skeletal ultrastructure; *Notosaria* is an impunctate Rhynchonellid (Jope, 1967) whereas

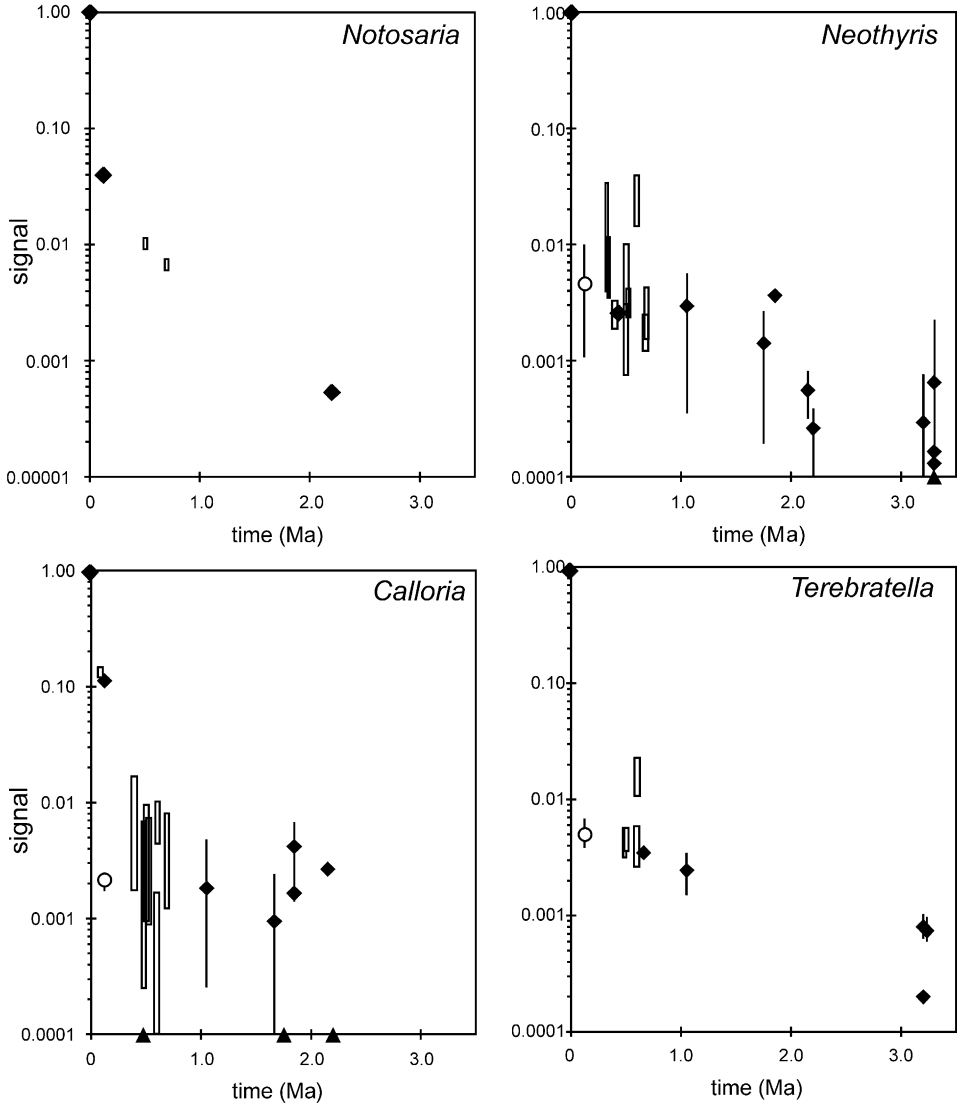

Fig. 1. Decline in immunological response in extracts from shells of four fossil genera. Where possible, dates from (Bowen et al., 1998) are used and represented by boxes, the width of which represents the isotope stage and the height of which represent ± 1 standard deviations (S.D.) from the mean (data given in Appendix). Other dates (◆, Walton, 1998) are less secure, and lines represent ± 1 S.D. from the mean where more than one sample was analysed. Samples which did not yield significant results (▲) and those from a probable reworked deposit identified by Bowen et al. (1998, ○) are also figured. The decline in total signal is reported for antisera relative to the nearest modern homologue.

the other three genera are punctate Terebratulids (MacKinnon and Williams, 1974). The ultrastructural differences are also reflected in biochemical variation of the respective shell matrices (Jope, 1967).

In previous studies of brachiopods from this succession, our criterion for accepting detected epitopes as original was one of systematic authenticity. We required the immunoreactivity of extracts of fossil shells to display a systematically meaningful pattern when tested with a range of polyclonal antisera raised against progressively more distantly related genera (Collins et al., 1991b). The same approach was used in this study. Despite the very rapid decline in overall signal, a pattern of reactivity at least partly consistent with the modern sample was obtained in material of less than 2 Ma (Fig. 2). The pattern of reactivity in older samples is different, and although this might be interpreted as the detection of phylogenetically uninformative epitopes, the weak signals involved are too close to background to allow such an inference to be made with confidence. Indeed a number of Pliocene samples did not display reactivity significantly above background.

The very similar rate and extent of the decay of immunoreactivity between the New Zealand genera prompted us to compare these results with a fifth genus from localities along the north western seaboard of the USA. This brachiopod, *Terebratulina*, belongs to an Order (Cancellothyridacea) not represented in the New Zealand material, but has a (punctate) skeletal ultrastructure similar to the Order Terebratulida. The decline in immunoreactivity corresponds closely with the New Zealand samples (data not shown). The conformity between samples is quite remarkable for this kind of analysis, where vagaries of diagenesis and the influence of microbial recycling lead to highly variable rates of decay.

3.2. Amino acid analysis

Full details of the amino acid analysis results are given elsewhere (Walton, 1998). The absolute abun-

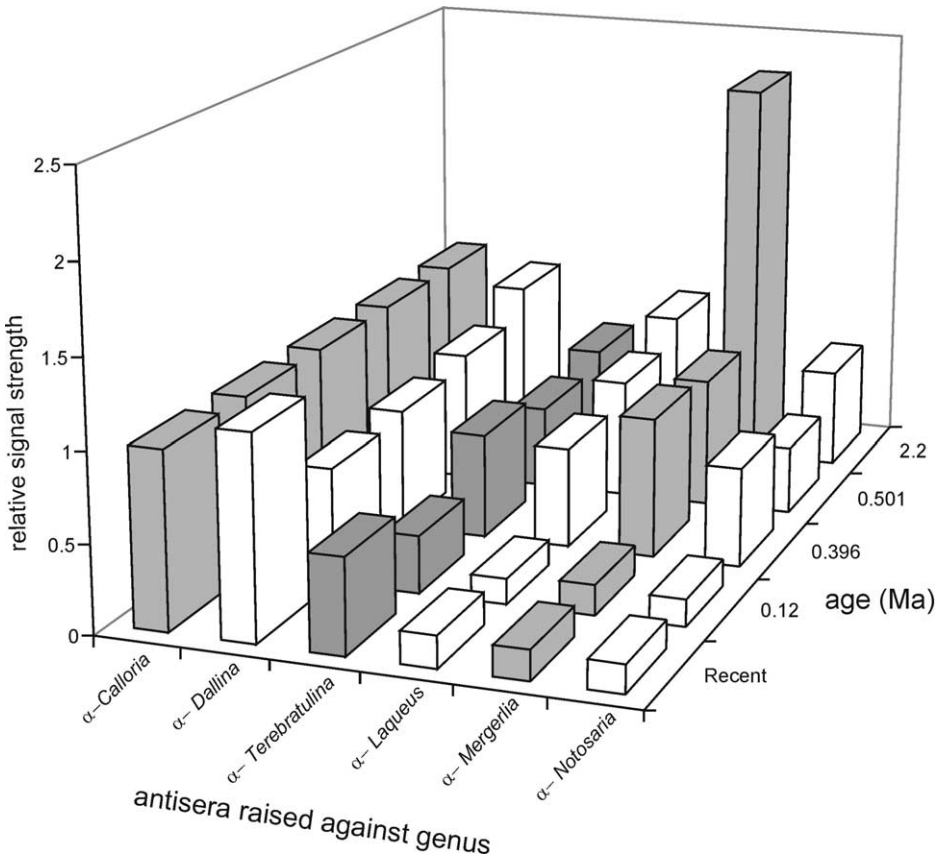

Fig. 2. Comparison of relative signal strength of different antisera (α-genus) reacted with samples of fossil *Calloria* of increasing age . In all but the oldest sample (2.2 Ma) signal strength is highest in antisera raised against the most closely related genus (*Dallina*). The pattern of cross-reactivity changes with time, and in the sample at 2.2 Ma, the strongest signal is from an antiserum raised against a distantly related genus (α-*Mergerlia*).

dance of amino acids (bound and free) decreases by about 50% over 3 Ma (Walton, 1998). The pattern of loss of peptide bonds is broadly similar to that of decay in the immunological signal (Fig. 3) with both showing a very rapid initial decline and a long persistent tail. However, in marked contrast to the low and persistently declining immunological signal, there is a significant residual fraction of bound amino acids (approximately 10%; Fig. 3). The persistence of peptide bound amino acids in all samples, despite the rapid increase in free amino acids is a striking feature of the data and echoes results with another apparently closed system, heated ostrich eggshell (Miller et al., 1992).

4. Discussion and conclusions

Immunological responses to a variety of extracts have been reported in the archaeological and geological record (Quarino and Koblenski, 1988; Muyzer et al., 1984, Muyzer et al., 1992a,b; Lowenstein et al., 1991; Schweitzer et al., 1997; Gibert et al., 1998), but this present study is the first to systematically investigate the pattern of decay. In contrast to previous reports of the decay of immunological response, at any one time the range of responses is small, and the overall pattern of decay is remarkable in its consistency (Fig. 3). The same pattern emerges irrespective of the brachiopod genus studied. In a preliminary investigation of mollusc shells (*Mercenaria*, Appendix 2) there is a suggestion of a similar pattern.

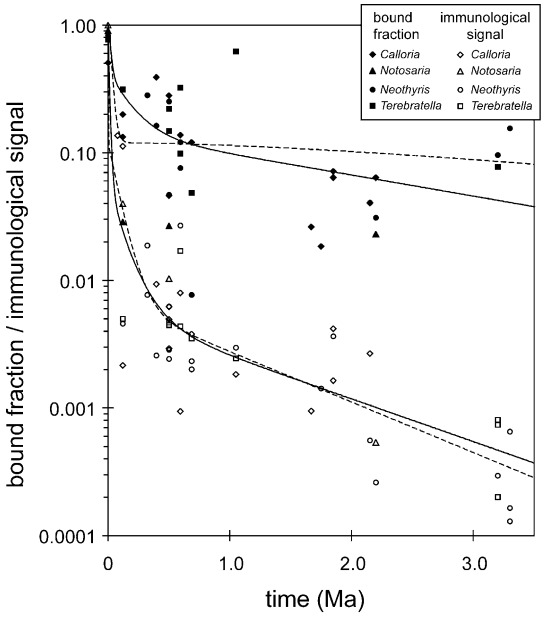

Fig. 3. Model fits to the bound amino acid fraction and immunological signal. The unbroken lines show the residual bound fraction and immunological signal for the three-rate model of hydrolysis. The broken lines show predictions of the three-pool model. For information on the parameters used see the Appendix.

Table 1
Parameters used in model fits to the decline in the bound amino acid fraction and the immunological signal (Fig. 3)

Model	Three pools	Three rates
Epitope size	45	3
k_1 (y^{-1})	4.7×10^{-5}	6.4×10^{-5}
k_2 (y^{-1})	2.5×10^{-7}	5.7×10^{-6}
k_3 (y^{-1})	2.1×10^{-8}	3.9×10^{-7}
λ_1 or μ_1	0.88	0.75
λ_2 or μ_2	0.11	0.17
λ_3 or μ_3	0.01	0.08

In the three-pool model, λ_i is the proportion of the number of complete protein molecules in the shell that resides in pool i. In the three-rate model, μ_i is the proportion of the number of bonds in each complete protein chain that are hydrolysed at the rate k_i. For details of the two models see the Appendix.

The most striking features of the immunological data are the very rapid initial decay of immunological signal and the very slow subsequent decline. Similar patterns have been reported in albumin from both pack- middens (Lowestein et al., 1991) and heated bone (Collins et al., 1998). This pattern is not observed for the mineral bound portion of osteocalcin, which approximately conforms to a first order rate law (Collins et al., 2000).

It is apparent from Figs. 1 and 3 that much younger fossils would have to be studied to determine the initial rate of decay in the immunological signal. In order to estimate the necessary age range of such samples, Arrhenius parameters were established from dry heating experiments of isolated secondary layer fibres (140–90 °C). The parameters derived from this study ($E_a = 116.4$ kJ mol^{-1}, $A = 1.15\times10^{10}$ s^{-1}) predict a half-life of 3 ka for the initial decay in immunological signal at 13.5 °C; present-day mean annual air temperatures (MAT) within the basin lie between 13.0 and 13.7 °C (Bowen et al., 1998). The rate predicted from the heating experiments is plausible, a similar rate has been reported for the decay of epitopes from albumin recovered from ancient pack-rat middens (Lowenstein et al., 1991 and Fig. 4). These results imply that it would be necessary to have access to Recent, well-dated sediment cores.

It is unclear why the pattern of decay in the immunological signal should display two such disparate rates. Polyclonal antisera were used in this study, which contain antibodies against a number of different epitopes from both carbohydrate and protein (Collins et al., 1991a). It is possible that the rapid decay of epitopes to higher order structure and/or those that include unstable amino acids (such as Ser or Asn) could contribute to the rapid initial decline in overall immunological signal.

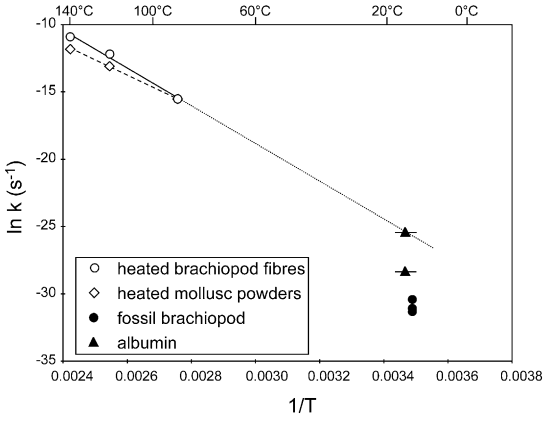

Fig. 4. Comparison of high temperature experiments with heated brachiopod fibres (○) and mollusc powders (◇), and rates observed for fossil brachiopods (●, MAT 13.5 °C) and albumin (▲). Albumin data from pack-rat middens at Falling Arches Utah, 37°24′ N, 109°38′ W (Lowenstein et al., 1991). Error bars represent upper and lower temperature estimates (specifically the effective surface temperature, 18.12 °C and MAT, 12.4 °C) which were derived from long-term temperature data, Bluff Weather station (37°28′ N 109°55′ W, December 1911–December 1987).

The apparently similar patterns of loss of amino acids and decay in the immunological signal were explored using a polymer decomposition model (Riley and Collins, 1994). Two models of hydrolysis based upon the assumption of random chain scission were constructed to predict the survival of the residual bound fraction of amino acids, and were then used to drive a simple model of the decline in immunological signal. In order to reproduce the initial rapid loss of amino acids and the subsequent persistence of a residual bound fraction, the models employ more than one rate of hydrolysis. The first hydrolysis model assumes the existence of three separate pools of protein, each characterised by a different rate of hydrolysis, whereas the second model is based on the assumption that all protein chains are essentially similar, but that the peptide bonds within them are hydrolysed at three different rates. Models with fewer than three rates of hydrolysis failed to provide adequate fits to the data. Immunological epitopes are modelled simply as contiguous residues of a given length, the number of which declines as the protein molecule degrades. Model fits to the data were achieved by numerically minimising the sum of the squares of the residuals in logarithm space. Details of the models are given in the Appendix.

The model based on three pools of protein does not strongly constrain epitope length; acceptable fits are possible with epitopes of between 14 and 45 residues. The three-rate model is very sensitive to the epitope length, which has to be equal to 3 to achieve a good fit. This length is, intriguingly, the minimum length size of a peptide epitope (e.g. Sela and Pecht, 1996). Unfortunately the use of polyclonal antisera against undefined antigens limits any detailed consideration of the relationship between the two measures of diagenesis. In order to investigate the phenomenon further, monoclonal antibodies against defined sequences would be required. One area for further investigation is the causes underlying the apparent dichotomy in rates of epitope diagenesis. At this stage, we are unable to determine whether the reduction in the rates of decomposition is due to (a) the absence of water for complete hydrolysis or (b) the presence of biological structures that are more resistant to chemical destruction.

The similar patterns of decomposition of both amino acids and immunological signals in each of the four genera support the view that brachiopod shell fibres represent a closed (or at least restricted), pH buffered system offering an 'exceptional' environment for preservation. Nevertheless survival to 0.5 Ma at an ambient temperature of 13 °C (Fig. 3) is only equivalent to approximately 4 Ma[1] in deep ocean sediments (2 °C). There is no evidence from this study to support claims for exceptional preservation of Mesozoic fossil proteins (Muyzer et al., 1992a, Schweitzer et al., 1997). In this respect, the closed system of brachiopod shells is apparently less promising that osteocalcin in bones (Collins et al., 2000).

Acknowledgements

Dr. John Wehmiller (University of Delaware) kindly supplied the fossil *Mercenaria* samples. The research was supported by grants from the Royal Society (MC), the Richard Lounsbery Foundation, New York (GM, PW) and NERC (MC). Northumbrian Biologicals are thanked for their generous donation of plastic-ware.

Associate Editor—**J.S. Sinninghe Damsté**

Appendix. Models of hydrolysis and decline of immunological response (epitope loss)

The models of hydrolysis are all based upon the concept of random chain scission of a polymer. We define α to be the probability that a peptide bond is broken by time t. The value of α depends upon the hydrolysis reaction kinetics, but for a first order reaction, which is assumed here, α is given by $\alpha = 1 - \exp[-kt]$, where k is the reaction constant.

A1. Residual bound fraction

Based upon the assumption of random chain scission, Riley and Collins (1994) give $\gamma_x(\alpha)$, the weight fraction

of x-mers in the polymer of initial length, P_0, at time t, as

$$\gamma_x(\alpha) = \begin{cases} \alpha \dfrac{x}{P_0}(1-\alpha)^{x-1}[2+(P_0-x-1)\alpha] & \text{for } x < P_0 \\ (1-\alpha)^{P_0-1} & \text{for } x = P_0 \end{cases} \quad \text{(A1)}$$

P_0 is taken to be 600 throughout (i.e. a shell matrix protein of ~60 kDa). The weight fraction of bound amino acids, γ_{bound}, is just one minus the weight fraction of free amino acids, and so

$$\gamma_{\text{bound}} = 1 - \gamma_1(\alpha) = 1 - \frac{\alpha}{P_0}[2+(P_0-2)\alpha] \quad \text{(A2)}$$

A2. Immunological response

We regard an epitope of length u to be any sequence of u residues with their connecting bonds intact, and so the number of epitopes of length u in an x-mer is

$$x - u + 1 \quad \text{(A3)}$$

It follows that the number of epitopes of length u contained in the x-mers present in a polymer is

$$(x-u+1)\frac{\gamma_x(\alpha)P_0}{x} \quad \text{(A4)}$$

and that the total number of epitopes of length u in a polymer is

$$\sum_{x=u}^{P_0}(x-u+1)\frac{\gamma_x(\alpha)P_0}{x} \quad \text{(A5)}$$

Substituting the expression for $\gamma_x(\alpha)$ [Eq. (A1)] in Eq. (A5) and dividing by P_0-u+1, gives $I(\alpha)$, the number of epitopes of length u in a polymer, expressed as a fraction of the initial number:

$$I(\alpha) = (1-\alpha)^{P_0-1} + \frac{1}{P_0-u+1}\sum_{x=u}^{P_0-1}(x-u+1)\,\alpha(1-\alpha)^{x-1}[2+(P_0-x-1)\alpha] \quad \text{(A6)}$$

A3. The three-pool model

In this model we assume that there are three distinct pools of protein. The protein in each pool is subject to random chain scission characterised by a pool-specific rate of hydrolysis, k_i, $i=1, 2, 3$. We define λ_i to be that proportion of the total number of complete protein molecules in the shell that resides in pool i. In this case the weight fraction of bound amino acids is given by

$$1 - \sum_{i=1}^{3}\lambda_i\gamma_1(\alpha_i) \quad \text{(A7)}$$

where $\alpha_i = 1-\exp[-k_i t]$ and the immunological response is

$$\sum_{i=1}^{3}\lambda_i I(\alpha_i) \quad \text{(A8)}$$

A4. The three-rate model

In this model we assume that all proteins behave identically, but that the peptide bonds are hydrolysed at three different rates, k_i, $i=1, 2, 3$. The proportion of the number of bonds in each complete protein chain that are hydrolised at the rate k_i, is defined to be μ_i. In the absence of detailed information about the positions of each type of bond in the molecule, it is assumed that they are randomly distributed. In this case the probability, $\bar{\alpha}$, that a bond chosen at random is broken at time t is given by

$$\bar{\alpha} = \sum_{i=1}^{3}\mu_i\alpha_i \quad \text{(A9)}$$

and the weight fraction of bound amino acids by

$$1 - \gamma_1(\bar{\alpha}) \quad \text{(A10)}$$

The immunological response is simply

$$I(\bar{\alpha}) \quad \text{(A11)}$$

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